Using the *Eurotium cristatum* Fungus for Preparing Fermented Herbal Teas

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Abstract: *Background*: The biological activities of dark Chinese teas are largely due to their microbial post-fermentation. Herbal teas are traditional Russian beverages that hold special value, owing to their taste and useful medicinal properties. However, no data are available in the literature on using microbial post-fermentation for enhancing their biological activity. The goal of this work was to demonstrate that the fungus *Eurotium* isolated from Chinese black teas can be used for the post-fermentation of herbal teas produced from bay willow and apple leaves.

Methods: *Eurotium cristatum* was isolated from brick Chinese tea Fujan and identified using conventional methods of microbiology and molecular biology. Low molecular weight metabolites (phenols, amines, sugars, and amino acids) were determined by HPLC. *E. cristatum* was grown in association with the bacterium *Bacillus amyloliquefaciens*.

Results: It was revealed to exhibit valuable biosynthetic features, such as a lack of mycotoxins, zero antimicrobial activity, and the capacity to synthesize neuroactive amines. *B. amyloliquefaciens* displayed a wide spectrum of antibiotic (antimicrobial and antifungal) activities that anifested themselves even with antibiotic-resistant bacteria). While growing on green unfermented tea (*Camellia sp.*) *E. cristatum* produced and modified neuroactive amines, such as dopamine, serotonin, and epinephrine. The fungus efficiently grew during the post-fermentation of herbal teas from both bay willow and apple leaves. Even though *Camellia* leaves substantially differed from bay willow and apple leaves in terms of phenol content, the growth of *E. cristatum* on *Camellia* was also sufficiently good. This suggests that the growth of *Eurotium* fungi is not influenced by the phenolic compounds. The data obtained on the composition of phenolic compounds, carbohydrates, and amino acids in the fermented plants and raw material provide evidence that the growth of the fungus proceeds depends on the hydrolysis of high molecular weight phenols and cell biopolymers in the fermented material.

Conclusion: Thus, the ability of *E. cristatum* to grow on plant leaves of significantly different biochemical composition provides foundations for new technologies aiming to obtain post-fermented herbal teas with high biological activity that are enriched in low molecular weight compounds including biogenic amines.

Keywords: Herbal teas, *Eurotium cristatum*, post-fermentation, biogenic amines, functional food, probiotics.

INTRODUCTION

It is widely accepted that tea is the most popular beverage in the present-day world. China is acknowledged as the main tea-producing and exporting country. Chinese tea varieties produced from *Camellia* sp. leaves are classified depending on their fermentation degree into (i) unfermented or partly fermented; (ii) fermented via spontaneous metabolic, predominantly oxidative, processes in tea leaves; and (iii) post-fermented by microorganisms that carry out the processes of autofermentation and autooxidation [1, 2]. Microorganisms accelerate the fermentation process in ripening teas so that it is accomplished within several months, in contrast to naturally maturating teas whose ripening takes several years.

Post-fermented teas are enriched in biologically active substances, and they contain not only tea components' autooxidation products but also

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substances resulting from their microbial transformation and microbial metabolites. There is much evidence that post-fermented teas ameliorate lipid metabolism, decrease the lipoprotein content of the total serum cholesterol and the low-density cholesterol fractions, and exhibit antimicrobial, antioxidant, and antimutagenic activity [3]. Such teas are beverages that are widely used in traditional medicine. For instance, Fuzhuan tea produces a significant antidysenteric effect [4]; it displays antimicrobial activity with a number of pathogenic microorganisms [5].

Post-fermented teas markedly differ from green (unfermented) and black (autofermented) teas in terms of chemical composition. Post-fermented teas are characterized by decreased catechin and α -theanine contents; they contain the catechin oxidation products theoflavins and thearubigins [3, 6].

Of special interest in terms of biological activity is the brick tea produced in the Hunan Province. This tea is obtained from *Camellia sinensis* leaves. The technology includes a microbial fermentation stage during which the brick tea becomes covered with gold brown spots called “golden flowers”. These “flowers” are the ascocarps of *Eurotium cristatum*, whose presence is a criterion of the standard quality of the *hei cha* brick tea. Research on the composition of the microbial community of post-fermented tea revealed that it includes fungi belonging to the genera *Penicillum* and *Sacharomyces*; besides, over 15 different fungal species of the genus *Eurotium* were identified [2, 3].

Studies with the fungus *E. cristatum* that dominates the *hei cha* (Fu Ten) tea revealed that it produces the alkaloids echinulin and cristatumins D. Echinulin exhibits antidiabetic activity, while cristatumins are characterized by moderate antimicrobial activity [7]. It was established that the aqueous extract of the fungus *E. cristatum* influences human lipid metabolism, inhibiting lipid accumulation in the cells. This enables considering *E. cristatum*-post-fermented teas as preventive medicine with respect to obesity development [8]. It was also demonstrated that the extracts of post-fermented *hei cha* tea produce a cytoprotective effect by protecting cells from oxidative stress; therefore, the extracts apply as a preventive drug for oncology disorders in the gut [3]. Apart from the research cited, there is much other evidence of the biological activity of post-fermented teas and their efficiency in preventing a large number of health problems.

It should be noted that, apart from consuming conventional tea produced from *Camellia sinensis* leaves, Russians have been using herbal tea from ancient times. Their production involves drying plant material, e.g., herbaceous plants and the leaves of fruit bushes and trees [9, 10]. No technological processes of microbial post-fermentation of herbal teas have been developed; such teas are not commercially available.

Importantly, representatives of the genus *Aspergillus* (the teleomorph of *Eurotium*) are detectable not only on tea leaves; they also occur in other plant species.

Therefore, the goal of the present work was to isolate the fungus *Eurotium cristatum* from post-fermented briquetted Chinese tea and to investigate its properties in relation to its potential use in the microbial fermentation of herbal teas produced from plant materials in central Russia. At this stage of our work, industrially produced herbal teas from bay willow and apple leaves (Moychay.ru) were used.

MATERIALS AND METHODS

Subjects

The microorganisms used in this work included a fungus identified as *Eurotium cristatum (*anam*. Aspergillus cristatus)* and a bacterium identified as *Bacillus amyloliquefaciens*; they were isolated from black brick tea named Fujian tea or Fu Tea (Fujian Province Guang Fu Tea Co., Ltd., Hunan Province, China) [11, 12].

To isolate the microorganisms that were contained in brick tea, a sample (10 g) of Fujian brick tea taken in the vicinity of a gold-brown spot was placed in sterile distilled water (100 mL), thoroughly stirred on a rotor shaker (200 rpm) for 20 minutes, and passed through a cotton filter; the filtrate was inoculated on Sabouraud agar. The plates were incubated at 24ºC for 7 days. The resulting individual fungal and bacterial colonies were reinoculated on agar plates at least 5 times. Subsequently, the isolated fungal and bacterial colonies that represented pure cultures were transferred to test tubes with Sabouraud medium slants for storage [13-15].

Culture Media and Cultivation Conditions

Five agar-containing media were used for cultivating the fungi and bacteria (composition indicated in %): (1) the Czapek medium: sucrose 3, $NaNO_3$ 0.3, KH_2PO_4 0.1, $MgSO_4 \cdot 7H_2O$ 0.05, KCl 0.05, $FeSO_4 \cdot 7H_2O$ 0.001, agar 1.5; (2) the Sabouraud agar: glucose 4, peptone

Table 1: Tested DNA Regions of the Fungus, Primers and PCR Annealing Temperature

DNA regions/genes	Symbols	Primers (5'→3')		PCR annealing temperature
		Symbols	Sequences	
Entire ITS rDNA and fragment of 28S rDNA	ITS-D1/D2	ITS1f	TCCGTAGGTGAACTTGCG	51ºC
		NL4	GGTCCGTGTTTCAAGG	
Large subunit 28S of ribosomal DNA	LSU	LR0R	ACCCGCTGAACTTAAGC	49ºC
		LR5	TCCTGAGGGAAACTTCG	
RNA polymerase II gene	RPB2	fRPB2-5F	GAYGAYMGWGATCAYTTYGG	From 60 to 50ºC
		fRPB2-7cR	CCAT(AG)GCTTG(CT)TT(AG)CCCAT	
Translation elongation factor 1-α gene	TEF1-α	EF-595F	CGTGACTTCATCAAGAACATG	From 66 to 56ºC
		EF-1567R	ACHGTRCCRATACCACCRATCTT	
β-tubulin gene	β-tub	βt2a	GGTAACCAAATCGGTGCTGCTTTC	From 64 to 54ºC
		βt2b	ACCCTCAGTGTAGTGACCCTTGGC	
Glycerol 3-phosphate dehydrogenase gene	GAPDH	gpd1	CAACGGCTTCGGTCGCA TTG	From 65 to 55ºC
		gpd2	GCCAAGCAGTTGGTTGTGC	

0.7, soy bean flour 0.3, yeast extract 0.4, agar 1.5; (3) the wort agar: wort 6 B, agar 1.5; (4) mineral agar Gause medium 1: soluble starch 2, K_2HPO_4 0.05, $MgSO_4$• $7H_2O$ - 0.05, KNO_3 0.1, NaCl - 0.05, $FeSO_4$• $7H_2O$ - 0.001, agar 2; distilled water, pH 7.2-7.4; (5) modified agar Gause medium 2: glucose 1, peptone 0.5, tryptone 0.3, NaCl 0.5, agar 2, tap water, pH 7.2–7.4.

The Sabouraud agar medium was used to store and maintain the fungal culture. The fungus was incubated at 24ºC for 4-14 days and stored at 4ºC; it was reinoculated once in every 2 months. Modified agar medium 2 was used for cultivating the bacteria. The bacterial culture was incubated at 24ºC for 7 days and stored at 4ºC it was reinoculated once in every 2 months.

Species Identification of the Fungus

Fungal isolates were identified according to their morphological and molecular genetic traits.

Spore formation, pigmentation and growth rate patterns were investigated on a number of different media for 4-21 days. The preparations were studied at a magnification of 150, 600, and 1500 using a Mikmed-6 light microscope (LOMO-Microanalysis, Russia).

The nucleotide sequences of fragments of ribosomal RNA genes and some other genes were compared with those available in databases. To compare the nucleotide sequences of related species, the genomic DNA of the tested strain was isolated using the DNeasy PowerSoil Kit (Qiagen Inc., USA) according to the manufacturer's instructions. The sequence of six regions of DNA was determined (Table **1**). The polymerase chain reaction (PCR) was carried out using a set of PCR Master Mix reagents (Thermo Scientific, USA).

The final volume of the PCR mixture of 50 μL included: 25 μL of 5X PCR Master Mix (ThermoScientific, USA), 0.5 μM of each primer, 1-100 ng of isolated DNA, and water (nuclease-free). (Extraction of genomic DNA from bacterial and fungal biomass was performed using DNeasy PowerSoil Kit (Qiagen Inc., USA). The universal bacterial primers 27F (AGA GTT TGA TCC TGG CTCAG) and 1492R (TAC GGY TAC CTT GTT ACG ACT T) were used to amplify the 16S rRNA gene [16]. The fungal primer sets used in this work are described in Table **1**. PCR was performed on a Thermal Cycler 2720 device (Applied Biosystems, USA) according to the thermo cycling programs.

PCR was conducted as follows:

- for ITS (1) 94ºC; 5 minutes, (2) 33 cycles with alternating periods with a temperature of 94ºC (1 minute), 51ºC (1 minute), 72ºC (1 minute), and (3) 72ºC (7 minutes);
- for LSU (1) 94ºC; 5 minutes, (2) 33 cycles with alternating periods with a temperature of 94ºC (1 minute), 51ºC (1 minute), 72ºC (2 minute), and (3) 72ºC (7 minutes);
- for regions 5-7 of RPB2 (1) 94ºC 5 minutes, (2) 9 cycles (the temperature decreases by 1 degree

upon transition from cycle to cycle) 94ºC; 1 minutes, 60 to 50ºC 1 minute, 72ºC 1.5 minutes, (3) 32 cycles at 94ºC 1 minute, 50ºC 1.5 minutes, 72ºC 1.5 minutes, (4) 72ºC 7 minutes;

- for TEF-1α (1) 94ºC; 5 minutes, (2) 9 cycles (the temperature decreases by 1 degree from cycle to cycle) 94ºC; 1 minute, 66ºC to 56ºC 1 minute, 72ºC 1.5 minutes, (3) 32 cycles 94ºC 1 minute, 56ºC 1.5 minutes, 72ºC 1.5 minutes, (4) 72ºC 7 minutes;

- for β-tub (1) 94ºC 5 minutes, (2) 9 cycles (the temperature decreases by 1 degree from cycle to cycle) 94ºC 1 minute, 64ºC to 54ºC 1 minute, 72ºC 1.5 minutes, (3) 32 cycles 94ºC 1 minute, 54ºC 1.5 minutes, 72ºC 1.5 minutes, (4) 72ºC 7 minutes;

- for GADPH (1) 94ºC 5 minutes, (2) 9 cycles (the temperature decreases by 1 degree from cycle to cycle) 94ºC; 1 minute, 65ºC to 55ºC 1 minute, 72ºC 15 minutes, (3) 32 cycles 94ºC 1 minute, 55ºC 1.5 minutes, 72ºC 1.5 minutes, (4) 72ºC 7 minutes.

The PCR products were purified using direct DNA reprecipitation under mild conditions (0.125 M ammonium acetate in 70% ethyl alcohol). The analysis of the PCR products was carred out by electrophoresis in agarose gel using Tris-borate buffer (TBE, 10X, Fermentas, Canada) at an electric field strength of 7.6 V/cm.

Sequencing was performed using the following primers: ITS1f; LR0R; LR5; fRPB2-5F; fRPB2-7cR; EF-595F; EF-1567R; βt2a; and gpd1.

The nucleotide sequences of the fragments obtained were determined by the Sanger method with an automatic Genetic Analyzer 3500 sequencer (Applied Biosystems, USA). The fragments' nucleotide sequences were scanned, edited, aligned, and stored using BioEdit v. 7.2.5 [17], Mega 6 [18], and DNAStar Lasergene SeqMan v.7.1.0 (DNASTAR Inc., Madison, WI, USA) software. Sequence aligning was performed using the nucleotide sequences of the GenBank [19], CBS [20], and RDP [21] databases.

Species Identification of the Bacterium

The species of the bacterium isolated was determined according to its morphological characteristics and the data obtained on the 16S rRNA gene sequences.

The isolation of the genomic DNA from the bacterial biomass was carried out using the PowerSoil DNA Kit (MO BIO, Carlsbad, CA, USA). The PCR of the 16S rRNA gene was performed using a set of PCR Master Mix reagents (contains Taq DNA polymerase; Thermo Scientific, Foster City, CA, USA) with the universal bacterial primers 27f (AGA GTT TGA TCC TGG CTCAG) and 1492r (TAC GGY TAC CTT GTT ACG ACT T).

The PCR procedure was performed with a Thermal Cycler 2720 device (Applied Biosystems, USA) according to the following protocol: (1) 94ºC, 5 min, (2) 30 cycles with temperatures of 94ºC 1 min, 51ºC 1 min, 72ºC 2 min, and (3) 72ºC 7 min. The nucleotide sequences were determined by the Sanger method with the automatic sequencer Genetic Analyzer 3500 (Applied Biosystems, Beverly, MA, USA) using the universal bacterial primers 27f, 341f (CCT ACG GGA GGC AGC AG), 1100r (GGG TTG CGC TCG TTG), and 1492r. The Mega 7 program was used to assemble the nucleotide sequences [22]. The sequences obtained were compared with the nucleotide sequences of the 16S rRNA gene of the bacterial type strains from the GenBank databases [19] and the Ribosomal Database Project [11, 23].

Determination of the Antibiotic Activity of the Isolated Microorganisms

The test microorganisms used for determining the antibiotic activity of the fungal (*Eurotium)* and bacterial (*Bacillus)* isolate were as follows: *Bacillus subtilis* ATCC 6633, *B. pumilus* NCTC 8241, *B. mycoides* 537, *Micrococcus luteus* NCTC 8340, *Leuconostoc mesenteroides* VKPM B-4177 (a strain resistant to VRLM, glycopeptide antibiotics of the vancomycin group), *Staphylococcus aureus* FDA 209P (a methycillin-sensitive strain, MSSA), *S. aureus* INA 00761 (a methycilln-resistant strain, MRSA), *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Comamonas terrigena* VKPM B-7571, *Aspergillus niger* INA 00760, and *Saccharomyces cerevisiae* RIA 259. The test strains of the bacteria and fungi were cultivated on the surface of petri plates on Gause agar 2. The test strains of *A. niger* INA 00760, *S. cerevisiae* RIA 259, and *L. mesenteroides* VKPM B-4177 were incubated at 28ºC. The other bacterial strains were grown at 37ºC.

For submerged cultivation of the fungal and bacterial isolates, the inoculum (the surface culture) was transferred with an inoculation loop to 750 mL Erlenmeyer flasks with 150 mL of Gause medium 2. It

was incubated on a rotor shaker (200 rpm) at 28ºC. The antibiotic activity of the culture liquid was determined on days 2, 4, 7, and 14 of fungal/bacterial growth by the agar diffusion method. 1 mL of culture liquid was placed in 9 mm wells. The level of antibiotic activity was estimated from the diameter of growth inhibition zones around the wells.

Preparing the Nutrient Media for Cultivating the *Eurotium cristatum* Fungus from Plant Material

The media for cultivating the fungus were prepared from herbal teas produced by the Moychay.ru Company; they contained fermented apple and bay willow leaves.

Surface cultivation of *Eurotium cristatum* was carried out on agar media containing (1) herbal tea extracts or (2) disintegrated biomass obtained from these teas. Disintegrated bay willow and apple leaves (10 g) were extracted with water (200 mL) for 60 minutes at a temperature of 50ºC.

Fermentation of Plant Material by the Fungus *Eurotium cristatum* INA 01267

Fermentation of bay willow or apple leaves-containing herbal teas (Moychay.ru) with *E. cristatum* was carried out by placing 20 g of disintegrated leaves with a humidity level of 20% in 500 cm^3 Erlenmeyer flasks, sterilizing them (1 atm, 40 minutes), and adding 2mL of fungal ascospore suspension.

The inoculum was obtained by growing *E. cristatum* INA 01267 on a Sabouraud medium slant in 40 mL test tubes at 30ºC for 10 days. The test tubes with the *E. cristatum* biomass obtained were supplemented with 15 mL of sterile water; the biomass was suspended with a sterile pipette. The resulting aqueous spore and mycelium suspensions were combined in a single sample that was used as Inoculums. It contained (2.5) x 10^5 CFU/mL. Hence, the inculated biomass contained 1 x 10^4 CFU/g of fungal inoculum. Fermentation of herbal teas with the *E. cristatum* fungus was carried out for 14 days at 30ºC with intermittent mixing.

Quantitative Determination of the Growth of the *Eurotium cristatum* Fungus (CFU/g) on Plant Substrates

Fungal growth intensity on various plant substrates was determined by inoculating suspension and dilution samples on agar as described in a methodological work [25]. The cultures were grown under aerobic conditions at 25ºC for 5 days, and the numbers of colonies of mycelial fungi with typical macro- and micromorphological features in 1 g samples were calculated [25].

Determination of the Biochemical Characteristics of Fermented Plant Material

The samples of fermented bay willow and apple leaf biomass were dried to a humidity level of 5% at a temperature of 50ºC; aqueous extracts of fermented biomass (1 g of biomass in 20 mL of water) were prepared at an extraction temperature of 80ºC; the procedure was performed for 20 minutes. It was followed by centrifugation (3000 g; 15 minutes).

Free amino acids in the aqueous extracts were determined by HPLC on an Agilent 1200 (USA) chromatographer with a diode array detector (DAD) and a Luna C18(2) chromatographic column (150 x 4,4 mm; 5 µm Phenomenex, USA) equipped with a trap column.

Phenolic compounds and organic acids were determined by HPLC on an Agilent 1200 chromatographer with a DAD and a Hypersit ODS C18 column (250 x 4,6 mm; 5 µm Thermo Fisher Scientific, USA).

Sugars were determined by HPLC on an Agilent 1200 chromatographer with a refractometric detector (RID) and a Luna NH2 100A column (250 x 4,6 mm; 5 µm Phenomenex, USA).

The content of low molecular weight metabolites in fermented plant biomass was expressed as mg/mL of aqueous extract [24].

Determination of Biogenic Amines during Plant Material Fermentation by *Eurotium cristatum* INA 01271

The *Eurotium cristatum* INA 01271 isolate was cultivated (a) in a liquid medium (unhopped barley wort, 7^0 B) at 28ºC for 5 days in batch culture and (b) on disintegrated moistened (5%) Sanhe Maofen green tea (Moychay.ru) for 14 days. Upon completing the cultivation stage, we prepared (a) the culture liquid by centrifugation of the submerged fungal culture (5000 g, 20 minutes) and (b) the extract of fungus-fermented tea (see 2.9). Biogenic amies were determined by HPLC on an isocratic chromatographer equipped with a PM-80 pump, an LC-4B electrochemical detector (Bioanalytical Systems, USA), and an YMC-Triart C18 chromatographic column (150 x 3,0 mm; 5 µm (YMC Co., Japan).

Determination of Mycotoxins Synthesized by the *Eurotium cristatum* INA 01271 Fungus

The *Eurotium cristatum* INA 01271 isolate was cultivated in liquid Czapek medium at 28ºC for 7 days in batch culture. The resulting culture was centrifuged (5000 g, 20 minutes). Mycotoxins were determined in the culture liquid by HPLC on a TermoFinnigan chromatographer with a DAD and a Hypersil™ BDS C18 chromatographic column (200 x 4.6 mm; 5 µm Thermo Fisher Scientific, USA) [25]. The lowest HPLC-detectable mycotoxin concentration was 0.003, 0.01, 0.2, and 0.2 mg/kg for aflatoxin, patulin, deoxynivalenol, and zearalenone, respectively [26, 27].

RESULTS

Isolation of Microorganisms from a Tea Briquette

Visual examination of post-fermented Fujian tea briquettes revealed clearly visible golden spots that represented *Erotium* growth points. The tea's name *Golden Flower* is due to the growth of this fungus [3]. Inoculation of water suspensions of brick tea typically resulted in combined growth of fungal colonies belonging to a single morphological type and bacterial colonies that also belonged to a single morphological type (Figure **1**). To sum up, a binary fungus–bacterium association was obtained. The bacterial and fungal cultures were separately reinoculated many times, and 10 isolates of each culture were selected for subsequent research. The microbiological purity of the isolates obtained was confirmed by doing at least 5 consecutive passages on Sabouraud and wort agar.

Identification of the Fungal Species

Several species of the genus *Eurotium* were described earlier; they were isolated from brick tea (Fujian tea [2]). The species of the fungal isolate was identified according to its morphological and cultural characteristics as well as the gene sequence data obtained.

The strain of fungus grows approximately equally well on the following organic media: the Czapek, Sabouraud, wort-agar, and modified agar Gause medium 2. It grows poorly on mineral agar Gause medium 1 (Figure **1**). On Czapek medium, the fungal colonies are olive-beige; subsequently, they become olive-brown and the bottom side of the colony and the

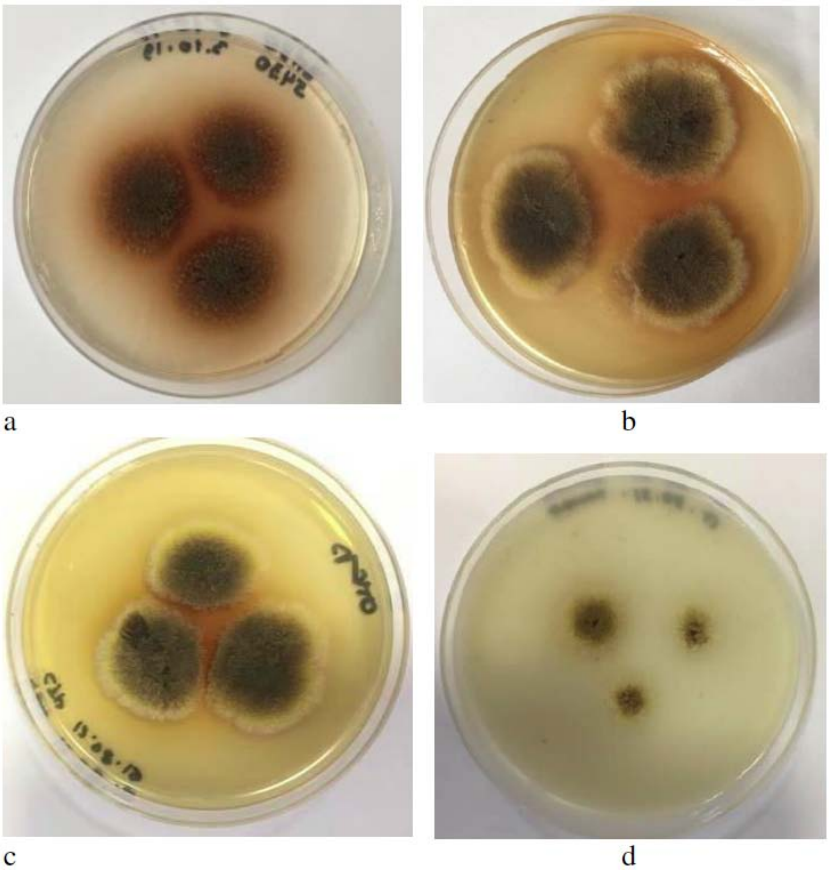

Figure 1: Colonies of the isolate *E.cristatum* INA01267 (7 days of growth at 28˚C) on agar media: (**a**) Czapek; (**b**) Sabouraud; (**c**) mineral Gause medium 1; (**d**) modified Gause medium 2.

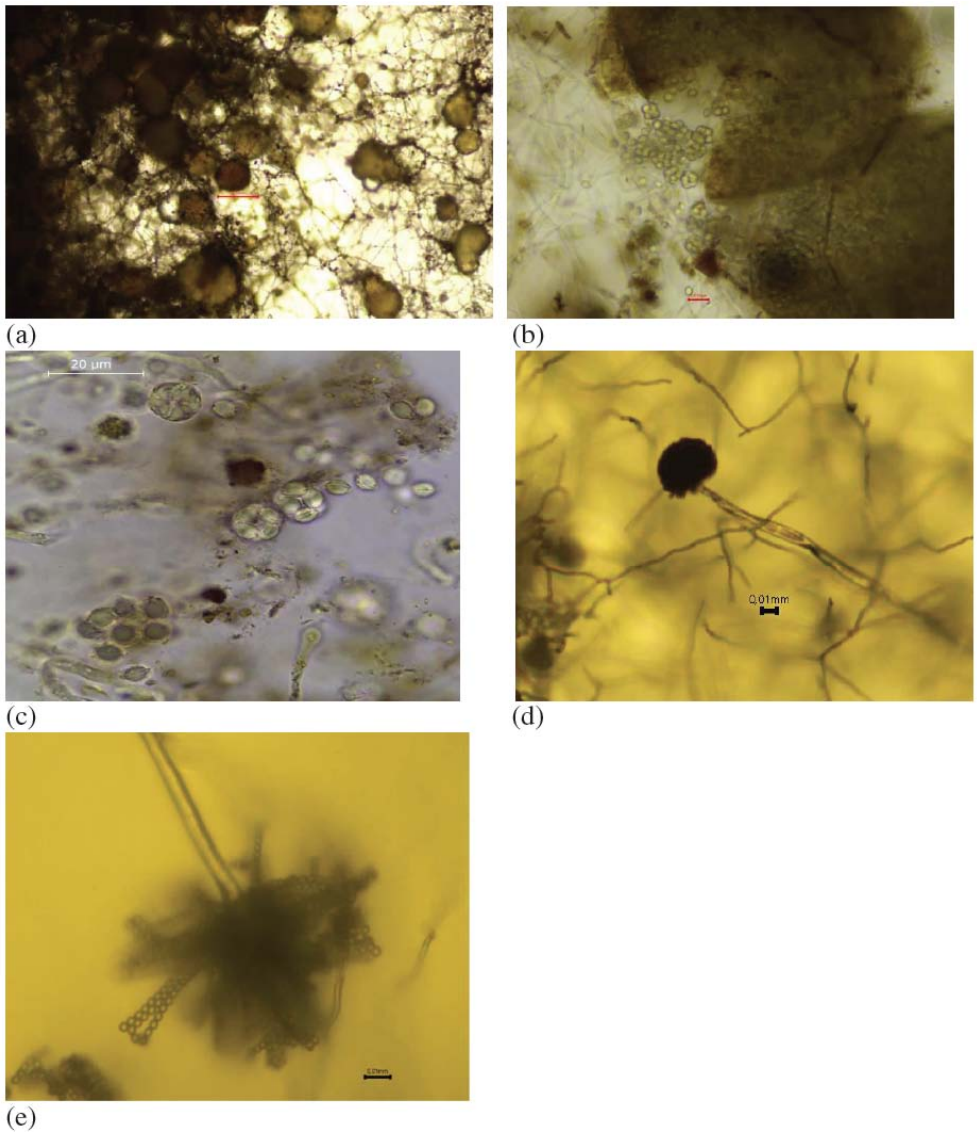

Figure 2: Stages of development of the fungus *E. cristatum* INA01267. Growth on Czapek agar: (**a**) fungal cleistothecia (4 days of growth); (**b**) asci extrusion from a broken cleistothecium (6 days of growth); (**c**) ascospores (7 days of growth). Growth on Czapek agar with 40% glucose; (**d**) conidiophores; (**e**) conidia.

exopigment turn reddish-brown; the growth rate is 5.2 mm/day. Abundant cleistothecia form on all agar media on day 4 of cultivation (Figure **2a**). The cleistothecium size was 45 to 108 µ. The asci that extrude from the cleistothecium (Figure **2b**) contain 8 ascospores each; the ascospores have prominent equatorial crests and a spiny, uneven surface (Figure **2c**). The spore size was 3.6 x 4.5 µ (Figure **4**). The growth intensity on the aforementioned media is the same if the fungus is grown at 37ºC, but no cleistothecia are generated. Conidia were only formed on Czapek medium with 40% glucose (Figure **2d**, **e**). The conidiophores were located under the cleistothecia-containing mycelium layer. The conidiophores: cleistothecia ratio was approximately 1:30. The conidium shape was globose to elliptical, their surface was smooth, and the size was 2.6 to 2.9 µ longitudinally. Taken together, the above criteria indicate that the fungus conforms with the description of the species *Eurotium cristatum* (Raper et Fennell) Malloch et Cain, teleomorph, or *Aspergillus cristatus* Raper et Fennell, anamorph [28, 29].

The sequences of six domains of the DNA of strain INA 01267 were determined (Table **2**). With four domains out of six (containing the ITS-D1/D2, RPB2, TEF1-α, and β-tub genes), a 99.67–100% match with the *Aspergillus cristatus* gene sequence in the databases was observed. This confirms the conclusion concerning the species identity of the isolate that was drawn from the morphological and cultural features.

The isolated strain was deposited in the Collection of the Gause Institute of New Antibiotics as *Eurotium cristatum (*anam*. Aspergillus cristatus)* INA 01267.

Table 2: Comparing the Nucleotide Sequences of the DNA Regions of the Strain *Eurotium cristatum* (anam. *Aspergillus cristatus)* INA 01267 with the DNA Sequences from the GenBank and CBS Databases

DNA regions/genes	Species*	Match (%)	Sequence length
ITS-D1/D2	*Aspergillus amstelodami* *Aspergillus cristatus* *Eurotium heterocaryoticum* *Aspergillus hollandicus* *Aspergillus montevidensis*	100	1079
LSU	*Aspergillus amstelodami* *Aspergillus glaucus* *Aspergillus montevidensis* *Aspergillus proliferans* *Eurotium herbariorum* *Eurotium rubrum* *Eurotium spiculosum*	100	848
RPB2	*Aspergillus cristatus*	100	997
TEF1-α	*Aspergillus chevalieri* *Aspergillus cristatus*	99.67	302
	Aspergillus niger	99.34	
β-tub	*Aspergillus cristatus*	100	395
GAPDH	*Aspergillus glaucus*	94.72	437

Identification of the Bacterial Isolate

The bacterial isolate (pure culture) grew quite well on agar-containing and liquid media, and its life-cycle culminated in endospore formation.

The cells of the tested bacterium were gram-positive and rod-shaped. Solitary cells, binary cell groups, or, more seldom, short chains (4–5 cells) were detected. The cell size was 3.6-4.5 x 0.5-0.7 μ. The endogenous spores were oval-shaped, their size was 1.0-1.4 × 0.5-0.6 μ. These features suggest that the tested strain belongs to the genus *Bacillus* [30]. It grows well on both synthetic and rich organic agar media. The growth temperature range is 28-37ºC. After 1 day of cultivation, the bacterium forms beige opaque colonies with a rounded edge and an elevated center on rich modified Gause medium 2, the colonies do not penetrate into the agar. Exopigment is lacking. At 37ºC, spores are produced starting from day 2 of growth; they account for at least 70% of the total cell number eventually. The bacterium is an obligate aerobe. In batch culture in a liquid medium, the bacterium forms a surface film. According to the sum of features, all the isolates were classified into the genus *Bacillus*.

The data obtained on the nucleotide sequences of the 16S rRNA gene enabled identifying the isolates as the species *Bacillus amyloliquefaciens* (Table **3**). Isolate 41 was deposited at the Collection of the Gause Institute of New Antibiotics as *Bacillus amyloliquefaciens* INA 01271.

Biological Activity of the Microbial Isolates Obtained from Briquetted Fujian Tea

Antibiotic Activity of the Isolates

In the first series of studies, the antimicrobial interactivity of the fungal and bacterial isolates was investigated. Antibiotic activity was determined in the culture liquid of the bacterium and the fungus grown in submerged culture in Gause medium 2 on days 2, 4, and 7 of cultivation. In addition, the antibiotic activity of the fungus was determined on days 14 and 21 of

Table 3: Comparing the Nucleotide Sequences of the 16srRNA Genes of Isolates 41 and 43 of *Bacillus amyloliquefaciens* with those Available from the Database

Bacterial clones	Species	Match; (%)	Sequence length
41	*Bacillus amyloliquefaciens*	100	1398
43	*Bacillus amyloliquefaciens*	99.5	1371

Table 4: Antimicrobial Activity of *Eurotium cristatum* INA 01267 and *Bacillus amyloliquefaciens* INA 01271

Cultural liquid of isolated from Fujian tea microorganisms	Days of growth	Growth inhibition areas, mm	
		***B. amyloliquefaciens* INA 01271**	***E. cristatum* INA 01267**
B. amyloliquefaciens INA 01271	2-7	-	34-24
E. cristatum INA 01267	2-21	0	-

cultivation (Table **4**). The *E. cristatum* isolate lacked antibiotic activity (data not shown), while the *B. amyloliquefaciens* isolate exhibited antifungal activity that reached the maximum at the beginning of the stationary phase (day 2 of growth); it decreased with culture aging, which was apparently due to the incapacitation of the active factor(s).

In the second series of studies, the spectrum of subjects tested for the antimicrobial effects of the bacterial isolate was enlarged: it included gram-positive and gram-negative bacteria (some of which were antibiotic-resistant) as well as mycelial fungi and yeast (Table **5**). The analysis of the culture liquid of strain *E. cristatum* INA 01267 revealed that it completety lacked antimicrobial activity. In contrast, the *B. amyloliquefaciens* 01271 isolate exhibited a wide spectrum of antibacterial and antifungal activities. It acted on the leuconostoc that is resistant to the antibiotics of the vancomycin group (strain VKPM B-4177) and on the methycillin-resistant strains of a *Staphylococcus* (strain FDA 209P – MSSA, strain INA 00761, MRSA).

Biogenic Amine Synthesis by E. cristatum INA 01267

It has been established that not only animals but also plants and microorganisms synthesize biogenic amines and their metabolites that impact the psychological state of humans. Such compounds are actively synthesized by lactic-acid bacteria that either form a part of the human microbiome or are plant symbionts. No similar data are available for fungi that are plant symbionts.

The synthesis of biogenic amines in *E. cristatum* was monitored by following their accumulation in the culture liquid while cultivating the fungus for 7 days in a liquid medium (wort). Concomitantly, biogenic amines were also determined in *Camellia* tea leaf extracts (Sanhe Maofen green tea, Moychay.ru) before and after fermenting them with the fungus *E*. c*ristatum*.

During the course of wort fermentation by *E. cristatum,* dopamine and serotonin were taken up from the medium, while their metabolites epinephrine and 5-HIAA acumulated (Table **6**).

Table 5: Spectrum of Antimicrobial Activity of *Bacillus amyloliquefaciens* INA 01271 (Culture Liquid; Growth on Gause Medium 2, 28˚C)

Cultural liquid of the strains	Days of growth	Growth inhibition areas, mm													
		***Staphylococcus aureus* INA 00761**	***S. aureus* FDA 209P**	***Bacillus subtilis* ATCC 6633**	***B. mycoides* 537**	***B. pumilus* NCTC 8241**	***Micrococcus luteus* NCTC 8340**	***Leuconostoc mesenteroides* VKPM B-4177**	***Mycobacterium smegmatis* VKPM Ac 1339**	***Mycobacterium smegmatis* mc^2 155**	***Escherichia coli* ATCC 25922**	***Pseudomonas aeruginosa* ATCC 27853**	***Comamonas terrigena* VKPM B-7571**	***Saccharomyces cerevisiae* RIA 259**	***Aspergillus niger* INA 00760**
INA 01271	2	24±	24±	14	16	15	14	0	0	12±	0	0	18	18	16
	4	22	18	12	20	20	24	0	26±	0	17	0	21	18	0
	7	14	16	10	12	12	15	14	0	0	21±	17±	13	17	17

Note: ±, growth inhibition.

Table 6: Biogenic Amine Content in the Culture Liquid of *E. cristatum* INA 01267

Biogenic amines / Sample	2,3-Dihydroxy phenyalanine (DOPA)	Dopamine (DA)	Norepinephrine (NE)	Epinephrine (E)	Dihydroxyphenylacetic acid (DOPAC)	3-metoxytyramine (3-MT)	Homovanillic acid (HVA)	Serotonin (5HT)	Dhydroxyindole acetic acid (5HIAA)
Wort	0.00	3570.15	0.00	0.00	0.00	88.00	236.48	915.29	329.24
E. cristatum-fermented wort*)	0.00	652.13	0.00	2470.98	0.00	0.00	15106.35	151.40	913.43
Tea[xx)]	1251.45	9567.56	0.00	0.00	962.02	0.00	857.01	523.13	2781.04
E. cristatum-fermented tea[xx)]	0.00	8.25	11.54	0.00	103.34	108.42	70.25	1458.94	36.56

*)the fungus was cultivated on unhopped barley wort (7° B), 14 days, 28°C
xx)the fungus was cultivated on disintegrated moistened (5%) Sanhzh Maofen green tea (Moychay.ru), 28°C, 14 days, water extraction (10 g in 200 mL of water).

Fermentation of *Camellia* tea also resulted in decreasing the dopamine content and increasing the amount of 3-metoxytyramine (3-MT), a product of dopamine oxidative degradation. Epinephrine was absent from the tea samples both before and after fermentation. The amount of serotonin that was present in the raw material was drastically increased after fermentation.

Mycotoxin Synthesis by E. cristatum

Some *Aspergillus* (teleomorph *Eurotium)* species synthesize mycotoxins during cultivation. The culture liquid after inoculating the *E. cristatum* INA 01267 isolate in liquid Czapek medium was tested for the presence of mycotoxins by HPLC.

The analysis of the data obtained (Table **7**) revealed that the fungus *Eurotium cristatum* virtually synthesizes no mycotoxins, and, therefore, it can be used for fermenting plant substrates in food industry.

Table 7: Mycotoxin-Producing Capacity of the Fungus *Eurotium cristatum* (Growth in Liquid Czapek Medium, 28°C, 7days)

Mycotoxin	Content in the tested sample, g/mL
Aflatoxin B_1	Below 0.003[x)]
Patulin	Below 0.01
Deoxynivalenol	Below 0.2
Zearalenone	Below 0.2

x)detection limits.

Fermenting Plant Substrates by *E. cristatum* INA 01267

Extensive studies have been conducted by Chinese researchers on the active growth of the fungus *E. cristatum* on *Caméllia sinénsis* tea leaves [2, 3, 6]. In an analogy to other post-fermented teas, active transformation of phenolic compounds proceeds during the microbial fermentation of *Caméllia* leaves-produced teas. In particular, there are data on the degradation of epigallocatechin-3-gallate (EGCG) and the formation of phenolic metabolites [31, 32]. These data along with other relevant information enable us to draw the conclusion that phenolic compounds are essential for the development of *Erotium*.

Research aimed at carrying out the *E. cristatum*-dependent postfermentation of plant materials traditionally used in Russia for preparing tea, such as bay willow (*Chamaenérion angustifolium*) and apple (Malus) leaves was the next stage of the present work. In terms of phenolic compound content, there is a significant difference between these plans and between each of them and *C. sinénsis*.

E. cristatum cultivation on agar media containing bay willow and apple extracts and disintegrated leaves revealed that the fungus grew sufficiently well in all tested media (data not shown).

If disintegrated sterile herbal tea leaves were fermented by *E. cristatum* INA 01267, its growth became visually detectable only on day 5 of cultivation. On day 14, the *E. cristatum* content on apple and bay willow leaves was 1.6 $x10^3$ CFU /g and 1.0 $x10^3$ CFU /g, respectively (Table **8**). Unfortunately, the quantitative determination of the *E. cristatum* content in fermented plant biomass is fraught with significant errors. Therefore, the intensity of its development was also estimated visually, based on counting characteristic golden spots. The visual data provided evidence that the *E. cristatum* content was 500 to

Table 8: Growth Intensity of *Eurotium cristatum* on Bay Willow and Apple Leaf, CFU/g

Fermented plant substrate	CFU/g		
	Fermentation day		
	5	10	14
Bay willow (*Chamaenérion angustifolium*)	$0.2*10^3$	$0.7*10^3$	$1.0*10^3$
Apple leaf (Malus)	$0.2*10^3$	$0.9*10^3$	$1.6*10^3$

1,000 colonies per g, i.e., 10 times higher on day 14 than on day 5.

Biochemical Characteristics of Fermented Substrates

Fermentation of plant substrates by *E. cristatum* was monitored by testing fermented herbal tea extracts for changes in the contents of phenolic compounds, sugars, organic acids, and amino acids.

Comparative analysis of the extracts revealed that the phenolic compound content in bay willow extact was considerably higher than that in apple extract (280 and 10.6 mg/dm^3, respectively). As the data of Table **9** demonstrate, apple leaf biomass fermentation resulted in producing 6.4 mg/dm^3 of gallic acid and eliminating such components as vanillin, syringaldehyde, and sinapic acid.

Bay willow biomass extracts were characterized by an increase in syringaldehyde and sinapic acid contents and a lack of sinapic and conyferyl aldehyde. Importantly, the total content of low molecular weight *phenolic compounds* did not significantly change after fermentation. The analysis of HPLC charts revealed a significant decrease in high molecular weight phenols (34—35 minutes) that was especially prominent with bay willow tea (Figure **3**).

The analysis of *carbohydrate* content in fermented tea extracts demonstrated that the fungus consumed sucrose and an insignificant fructose amount during bay willow tea fermentation. Apple leaf fermentation was also associated with sucrose and fructose consumption. The glucose levels only insignificantly changed in both tested systems (Table **10**).

However, the composition of *organic acids* that result from incomplete carbohydrate oxidation underwent appreciable changes (Table **11**). During fermentation, the tartaric and malic acid content substantially decreased, whereas the succinic acid increased. Succinic acid is an antioxidant that exhibits a wide spectrum of biological activities: it represents an adaptogen, improves the immunity system, and ameliorates the state of the cardio-vascular system of the organism.

Amino acid analysis before and after fermentation (Table **12**, **13**) revealed the presence of all essential amino acids in bay willow and apple leaf extracts. Interestingly, the fermentation of both substrates results in an increase in glutamine, asparagine, glycine,

Table 9: Influence of Bay Willow and Apple Leaf Fermentation on the Composition of Phenolic Compounds (Aqueous Leaf Extracts: 10 g in 200 mL of Water)

Phenolic compound (g/mL)	Bay willow		Apple leaf	
	Before fermentation	After fermentation	Before fermentation	After fermentation
Gallic acid	258.0	= 261.8 [x)]	0.00	> 6.40
Syringic acid	3.77	< 2.51	2.14	< 1.73
Vanillic acid	3.076	< 2.02	1.31	= 1.36
Vanillin	3.7	= 3.71	2.72	>0.00
Syringaldehyde	1.77	> 3.13	1.67	< 0.00
Sinapic acid	4.17	> 8.81	0.00	= 0.00
Conyferyl aldehyde	1.91	< 0.00	1.14	= 1.21
Sinapic aldehyde	2.65	<0.00	1.57	>1.93
Total	279.05	281.98	10.55	12.63

[x)]Symbols: = content unchanged; > content increased; < content decreased. The Note applies to Tables **9-13**.

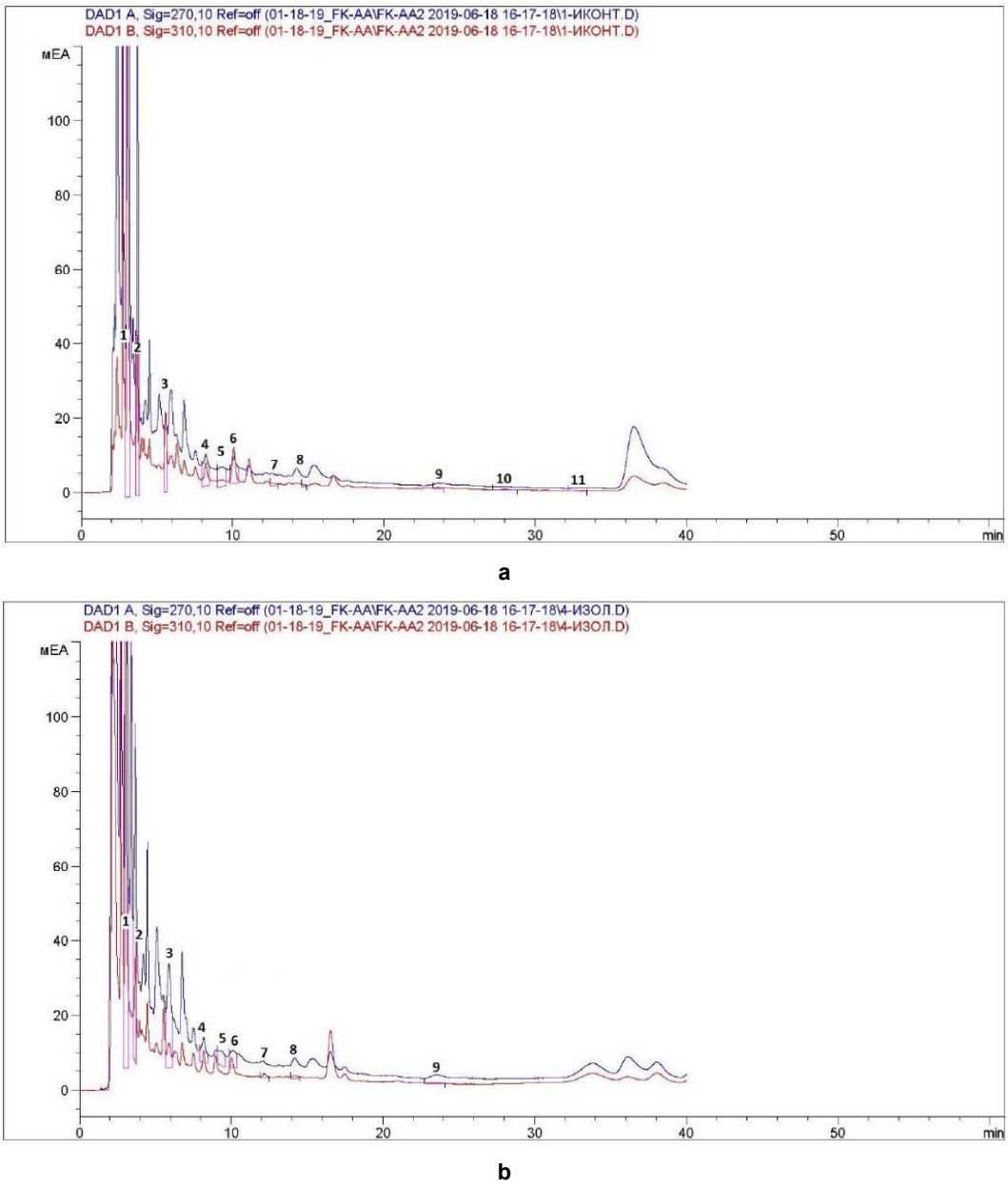

Figure 3: HPLC profile of the composition of phenolic compounds in bay willow extracts (**a**) before and (**b**) after fermentation by the fungus *E. cristatum* INA01267.

Table 10: Influence of Bay Willow and Apple Leaf Fermentation on Sugar Composition (Aqueous Leaf Extracts; 10 g per 200 mL of Water)

Sugar (mg/dm^3)	Substrate			
	Bay willow		Apple leaves	
	Before fermentation	After fermentation	Before fermentation	After fermentation
Glucose	2.46	= 2.48 [x)]	2.95	=2.75
Fructose	4.46	>3.95	3.30	>0.00
Sucrose	0.32	>0.00	1.04	>0.00
Total	7.24	6.43	7.29	2.75

Table 11: Influence of Bay Willow and Apple Leaf Fermentation on Organic Acid Content (Aqueous Leaf Extracts; 10 g per 200 mL of Water)

Organic acids (mg/dm^3)	Substrate			
	Bay willow		Apple leaves	
	Before fermentation	After fermentation	Before fermentation	After fermentation
Oxalic acid	1.67	1.62	1.38	1.67
Tartaric acid	9.65	2.29	5.77	0.00
Malic acid	2.58	1.95	1.27	0.00
Lactic acid	1.00	5.14	7.04	0.00
Citric acid	2.95	4.52	9.72	3.29
Succinic acid	2.32	7.70	2.80	4.23

Table 12: Amino Acid Composition of Bay Willow Leaf Extracts before and after Fermentation (Aqueous Leaf Extracts: 10 g per 200 mL of Water)

Peak No.	Content before fermentation, mg/L	Content after fermentation, mg/L	Compound
1	6.54	<4.51	Aspartic acid
2	0.814	>1.35	Glutamic acid
3	0.523	>0.905	Asparagine
4	0.526	=0.623	Histidine
5	0.658	>0.964	Serine
6	0.364	>>1.51	Glutamine
7	0.169	=0.230	Arginine
8	0.423	>0.925	Glycine
9	1.92	=1.46	Threonine
10	8.88	=7.45	Alanine
11	0.564	=0.665	Tyrosine
12	1.32	>1.68	Valine
13	0.333	=0.433	Methionine
14	0.896	=0.782	Tryptophan
15	1.08	=0.885	Isoleucine
16	1.11	=0.809	Phenylalanine
17	0.862	=0.897	Leucine
18	0.462	=0.569	Lysine

and valine contents. Prior to fermentation, apple leaf extracts (Table **13**) contained less aspartic acid, threonine, and alanine than bay willow extracts (Table **12**). An inconsiderable increase in aspartic acid and alanine content was detected after fermentation.

DISCUSSION

In food microbiology, much attention is paid to microbial contaminants involved in spoiling food with low Aw values. Special importance is placed on fungal contaminants, which is due to their capacity to produce toxins causing serious food poisoning. However, apart from toxigenic fungi, food products with low Aw values are efficiently utilized by non-toxigenic strains of *Aspergillus* (the telemorph of *Eurotium*), *Penicillium, Saccharomyces,* etc. Non-mycotoxigenic *Erotium* strains *(E.repens* and *E.rubrum*) are used in Southeast Asia as starter cultures for fermented fish products [33]. Special attention has been given to the species *E. cristatum* that develops on *C. sinensis* tea leaves. It is

Table 13: Amino Acid Composition of Apple Leaf Extracts before and after Fermentation (Aqueous Leaf Extracts: 10 g per 200 mL of Water)

Peak No.	Content before fermentation, mg/L	Content after fermentation, mg/L	Compound
1	1.48	>1.89	Aspartic acid
2	1.26	=0.938	Glutamic acid
3	0.623	>1.02	Asparagine
4	0.660	0.457	Histidine
5	1.23	=1.27	Serine
6	0.662	>1.07	Glutamine
7	0.192	=0.174	Arginine
8	0.947	>1.32	Glycine
9	0.716	=0.610	Threonine
10	1.89	>2.22	Alanine
11	1.32	<0.875	Tyrosine
12	0.836	>1.07	Valine
13	0.610	=0.438	Methionine
14	1.21	=1.10	Tryptophan
15	1.10	=1.06	Isoleucine
16	1.28	=1.06	Phenylalanine
17	0.930	=0.960	Leucine
18	0.393	=0.439	Lysine

widely used in China in industrial technology aimed at obtaining post-fermented tea varieties and represents an important factor affecting their quality, taste, and flavor [3].

In compliance with the goal of obtaining post-fermented Russian herbal teas, this work was concerned with a fungal isolate from dark briquetted Chinese tea that is referred to as the *Golden Flower* in the literature [2, 3].

Microbial Contaminants of Dark Chinese Tea

The brick tea (China, Fundzhuan) used by us to isolate *Erotium* strains was covered with a large number of “yellow spots” that represented the asci of fungi belonging to the genus *Erotium.* Inoculation of the “yellow spots” on agar-containing media typically resulted in the development of microbial associations. White colonies that formed on golden fungal mycelium were surrounded by a lysis area, indicative of the antibiotic activity of the developing microorganisms. The microbial association isolated by us represented a binary fungus–bacterium culture that efficiently developed on both nutrient-rich and mineral agar media.

In conformity with the description of the morphological properties of *Eurotium* fungi in the literature, these asomycetes formed bright yellow cleistothecia with smooth clear edges, red, orange, or brown hyphae, and pale yellow ascospores when cultivated on solid media. All *Eurotium* species are xerophiles, and their best growth and spore formation is attained on media with low Aw values. The isolate obtained in the present work fully conformed with the description of the *Eurotium* teleomorph, according to its morphological characteristics (Figures **2-4**). Comparison of the nucleotide sequences of the isolated strain with those of the ribosomal and genomic DNA from the Blast and CBS databases enabled, with 99 and 67-100% match for the ITS, RPB2, β-tub, and TEF1-α genes, classifying the fungal isolate into the species *Eurotium cristatum* (strain INA01267; Table **2**).

On the basis of comparing the nucleotide sequences of the 16S rRNA gene (Table **3**), the bacterial isolate was identified as *Bacillus amyloliquefaciens* INA01267 (Collection of Microorganisms, Institute of New Antibiotics, Russian Academy of Sciences).

These results support the suggestion that the *E. cristatum* fungus is the most widespread contaminant

associated with Chinese brick Fuzhuan tea [2, 31]. This is the first report on a binary fungus (*E. cristatum*)–bacterium association developing on tea leaves.

Importantly, a microbial consortium possesses practical advantages over separate cultures because it utilizes a broadened spectrum of substrates and produces an enlarged array of biologically active metabolites that can perform various functions.

Biological Activity of the Microbial Contaminants of Brick Tea

There is extensive literature on the biological activity of post-fermented dark Chinese teas. It mainly deals with the effects of tea varieties on human health and, therefore, with the contents and modes of action of anthraquinone pigments [34], catechins [3, 35], and phenolic compounds [31] as well as with the lipid content-decreasing effects of teas [36, 37] and their antimicrobial [38, 39], antioxidant [40-42], and antimutagenic activities [3].

Antimicrobial Activity of Microbial Isolates from Dark Brick Tea

The antimicrobial activity of tea extracts was demonstrated, in many studies, with respect to pathogenic bacteria such as *Salmonella typhimurium* [43], *Staphylococcus aureus,* and *Bacillus subtilis*, but not *Escherichia coli* [44]. The main antimicrobial agents singled out by researchers include plant polyphenols [38], epicatechin, and caffeine that presumably impact the activity of various enzymes and the stability of the membranes of pathogenic bacteria [3]. The putative mechanism of the antimicrobial activity of Chinese tea is based on our knowledge of the properties of phenolic compounds such as alkylresorcinols [45-47]. No data have been presented on the antibiotic activity of the microbial contaminants of *Camillia*.

Since several attempts to inoculate the "yellow spots" of brick tea typically resulted in producing a binary fungus–bacterium association, the bacterial cocontaminant seems likely to protect both the fungus and the plant from extraneous, especially phytopathogenic, microbiota. This suggestion was confirmed by the data on the antibiotic activity spectrum of the strains isolated by us. While the fungal isolate *E. cristatum* vitually lacked antibiotic activity, the bacterial isolate *B. amyloliquefaciens* exhibited a wide range of antibacterial and antifungal activities (Table **5**). The results obtained in the present work do not contradict literature data and actually supplement them.

Hence, the Chinese brick tea-isolated binary ascomycete–bacillum association can be hypothesized to play the role of the *Camellia* exosymbiont and to protect the host plant from microorganisms. Further studies with phytopathogenic microorganisms as test subjects can be conducted to verify this hypothesis. However, it seems more likely that the bacterial contaminant protects the fungus *Erotium* from extraneous microbiota. The benefit gained by the bacterium in this association is apparently based on its trophic interaction with the fungus that supplies low molecular weight nutrients.

An additional function that is plausibly performed by bacterial antibiotics is inducing spore formation. This kind of biological activity does not involve agonistic interaction. Instead, the following message is communicated regarding the associants' development stage: *spore formation is to be initiated*. The functions of antibiotics as "informobiotics" has been actively researched recently [48].

Food Safety of the Isolate *E. cristatum*

A prerequisite for using the fungal isolate *E.cristatum* INA01267 in terms of fermented herbal tea production is a lack of toxigenicity. The HPLC of the culture liquid of the fungus *E. cristatum* confirmed that it virtually lacked aflatoxin B1, patulin, and deoxynivalenol (Table **7**) that are produced by the toxigenic fungi of the genus *Aspergillus*.

Synthesis of Biogenic Amins by *E. cristatum*

The capacity to synthesize biogenic amines is apparently characteristic of all flora and fauna representatives. Some neurochemicals perform communicative and regulatory functions in diverse taxa of animals, plants, fungi, protozoans, and bacteria [50-57, 59], which enables using the more general term biomediators [58, 59].

Synthesis of neuroactive compounds by microorganisms forms a part of microbial endocrinology, an interdisciplinary area of research that strands the boundary between microbiology and neurology and focuses on neurochemical agents that are identical, homologous, or functionally analogous in the host organism and the microbiota "Microbial endocrinology is defined as the study of the ability of microorganisms to both produce and recognize neurochemicals that originate either within the microorganisms themselves or within the host they inhabit" [52]. Microbial endocrinology actually

emphasizes the fact that neuroactive substances formed both by multicellular organisms and microorganisms consititute an “universal language” that enables communication between different kingdoms and empires of life [60].

The evolutionarily primary role of neurochemicals and hormones could be that of microbial communicative signals, according to the hypothesis that eukaryotic communication agents evolved as a result of horizontal gene transfer from prokaryotic microorganisms [52, 61]. As pointed out in [52], the ubiquitous occurrence of neuroendocrine hormones in nonmammal biological systems suggests that their presence in mammal organisms is to be interpreted in terms of their evolutionary prehistory.

In conformity with the principles of microbial endocrinology, it was established that a large number of microorganisms, including pathogens and the symbiotic microbiota of mammals, produce neuroactive compounds, such as biogenic amines [28]. Synthesis of dopamine, norepinephrine, serotonin, and other neurochemicals was revealed in plant microsymbionts that form a part of starter cultures used for producing fermented dairy products [48].

In this work, it was demonstrated for the first time that *Eurotium* fungi synthesize neuroactive compounds and metabolize biogenic amines contained in plant substrates during their fermentation (Table **6**).

Wort (used as growth substrate) contained high concentrations of serotonin (5-HT), its degradation product 5-dihydroxyndoleacetic acid (5-HIAA), dopamine (DA), and its degradation products 3-metoxytyramine (3-MT) and homovanilic acid (HVA). Wort fermentation by *E.cristatum* resulted in considerably decreasing its serotonin and dopamine contents and concomitantly increasing the HVA content. *E. cristatum* cultivation enriched the wort medium with another neurochemical, epinephrine.

The *Camellia* tea extract was characterized by a significantly elevated biogenic amine content, compared to the wort (Table **6**). The tea extract contained DA, 5-HT, the dopamine precursor 2,3-dihydroxyphenylalanine (DOPA), its degradation products dihydroxyphenylacetic acid (DOPAC) and HVA, and 5-HIAA.

Fermenting tea leaves by *E.cristatum* resulted in changing the biogenic amine compostion of the leaf extract. The 5-HT content drastically increased, due to inhibiting its oxidation to 5-HIAA by the fungus. The DA content decreased. The products of metabolizing DA by the fungus, such as 3-MT and HVA, were also detected. Hence, *E. cristatum* took up dopamine from the medium, irrespective of its composition and cultivation conditions. It synthesized serotonin and epinephrine when cultivated on tea and wort, respectively.

In Figure **4**, the metabolic pathways of biogenic amines during the fermentation of wort (Figure **4a**) and green unfermented tea (Figure **4b**) are shown.

DA is converted to epinephrine (E) and oxidized to HVA during wort fermentation. However, the DA → E conversion pathway does not play an important role during tea fermentation, and DA is oxidized to HVA via 3MT and DOPAC. Tyrosine is predominantly oxidized to 5-HIAA.

During tea leaf fermentation, 5-HT is synthesized from 5-hydroxytryptophan (5-HTP) in amounts that are three times larger than those contained in tea before its fermentation by *E. cristatum.* Importantly, the synthetic and transforming activities of the fungus *E. cristatum* with respect to biogenic amines was revealed for the first time in this work. Wort fermentation by the fungus predominantly activates the dopamine oxidation and epinephrine synthesis pathway, whereas tea fermentation involves the serotonin synthesis pathway.

Herbal Tea Fermentation by *E. cristatum*

Since *E. cristatum* predominantly inhabits the soil and plant leaves in nature, it seems likely that it can develop not only on *Camellia* leaves, but also on other plant substrates. This capacity of the fungus was confirmed in our studies with herbal teas that are industrially produced in Russia.

Apart from tea that is traditionally consumed in many countries and obtained from *Camellia sinensis* leaves, there are other beverages produced from leaves and herbaceous plants. Among such herbal teas, mate (*Ilex paraguariensis*), hibiscus tea (*Hibiscus sabdariffa*), peppermint tea (*Mentha piperita*), and camomile tea (*Matricaria recutita*) are particularly widely spread. These beverages are routinely consumed in various regions of the world for medical purposes, to quench thirst, and for pleasure. The majority of these plants are characterized by high contents of phenolic compounds, antioxidants, and compounds that exert a psychological influence on humans.

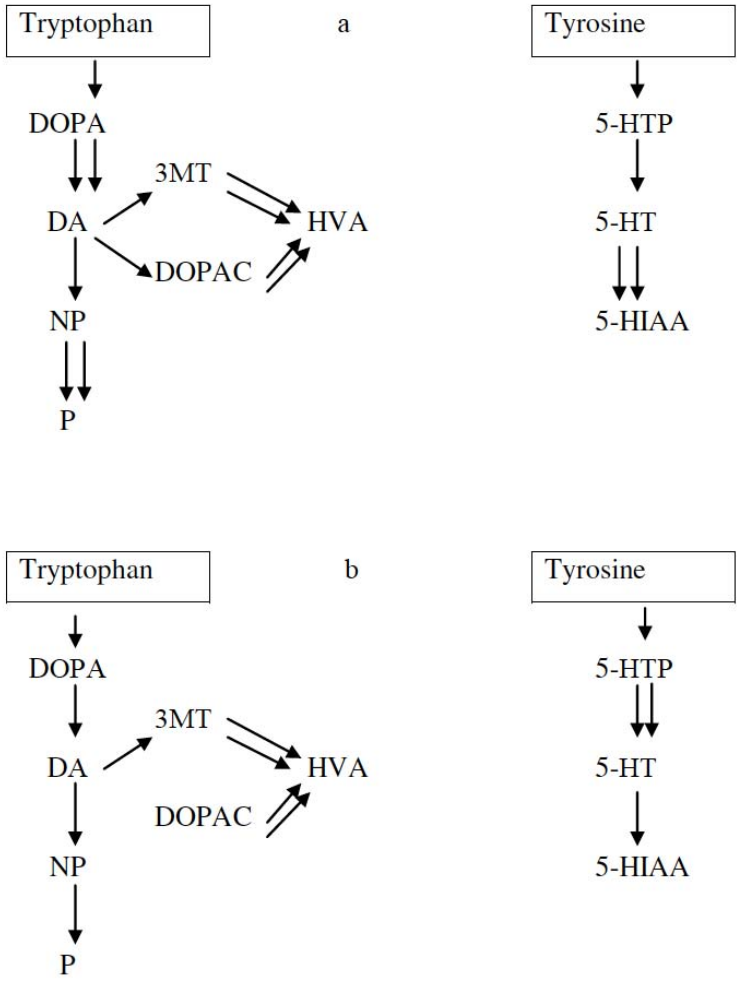

Figure 4: Metabolic pathways of biogenic amine conversion during wort (**a**) and *Sanche Maofen* green unfermented tea (**b**) fermentation by the fungus *E.cristatum* INA01267. See text for designations.

Recent research has revealed that many plants used for producing herbal teas can reduce body weight, prevent the development of oncology disorders, and produce antibacterial, anti-inflammatory, spasmolytic, and restorative effects on the human organism. The ability of flavonoid-containing plant extracts to prevent or ameliorate metabolic disorders associated with type 2 diabetes has been confirmed in clinical studies [62].

The recent increase in demand for healthy food has promoted research aimed at producing herbal teas [9]. Plants contain biologically active substances, including vitamins and vitamin-like compounds, micro- and macroelements, phenolic compounds, and organic acids. Supplementing traditional tea varieties with them enables enhancing their functional value [10].

Beverages with good organoleptic properties and high antioxidant activity were obtained from the black currant, raspberry, briar, mountain ash, snowball, marjoram, and thyme leaves. A project currently in progress is aimed at employing novel plant raw materials and secondary plant resources, including fruit plant leaves and herbs, for producing functional beverages [10].

In our studies, bay willow and apple leaves that were obtained from a herbaceous plant and a tree, respectively, were chosen as raw materials for obtaining fermented herbal tea. Dry tea leaves obtained from these plants by Moychay.ru were subjected to fermentation.

The phenolic compounds of by willow leaves are dominated by tannins exemplified by oenothein; flavonoids (quercetin, mericitin, quercitrin and kaempferol), and quercitrin-galloyl-galactoside and such phenols as gallic acid and ellagic acid are present at lower concentrations. The content of total phenols and tannins in bay willow leaves is 300 and 200 mg/g of dry leaves, respectively [10]. During oxidative fermentation (not involving microorganisms), the composition of the phenolic compounds undergoes

significant changes. There is an increase in the content of phenolic acids and flavonoids that exhibit high antioxidant activity. Compounds with hydroxyl groups in the ortho position such as quercetine are highly active antioxidants. Oxidized phenolic compounds can be condensed to form tannin or reduced via interaction with other available antioxidants. Less information is available on the composition and transformation of phenols in apple leaves than in bay willow leaves. Nevertheless, there are common general patterns in the relevant processes in both plants [9].

The impact of microbial fermentation (post-fermentation) on the composition of phenolic compounds has been studied in detail in *C. sinensis* leaves. Oxidation of gallocatechin galate (GCG) and epigalocatechin galate (EGCG) contained in tea leaves results in formation of their condensation products, such as theoflavins and theorubigines. Secondary fermentation by *Erotium* fungi that is carried out to produce Puerh tea results in decreasing the content of complex phenolic compounds, including EGCG and GCG, and increasing the amounts of simple catechins (unrelated to gallic acid), free gallic acid, and the products of its transformation [31, 32]. The amounts of all these compounds also decrease during the course of long-term fermentation. Hence, the fungus degrades complex phenolic compounds during the secondary microbial fermentation of *Camellia* teas. Degradation products accumulate in the leaves. Subsequently, they are partly assimilated by the fungus [3, 63].

Analogous processes are carried out during *E. cristatum*-dependent fermentation of the leaves of other plants, exemplified by the bay willow and the apple. An increase in gallic acid content in leaf extract upon fungal fermentation testifies to tannin hydrolysis. Redox processes associated with interactions between antioxidants result in changing the aldehydes–acids ratio (Table **9**). The syringic and vanillic acid contents in fermented bay willow leaf extracts decrease, and the syringaldehyde content increases, while the vanillin content does not change. Sinapic and conyferyl aldehyde are completely eliminated, whereas the sinapic acid content increases.

The free gallic acid content considerably increases in apple leaves that contain less phenols; vanillin and syringaldehyde disappear; the conyferyl and synaptic aldehyde contents insignificantly increase, and the syringic acid content slightly decreases.

To sum up, the secondary microbial fermentation of plant material involves the hydrolysis of complex phenols and further oxidative processes (Figure **3**, Table **9**). Degradation of complex phenols in bay willow leaves is illustrated in Figure **3a** and **b**. It is evident that the high molecular weight phenol peak does not show. The results obtained are consistent with the data on the degradation of complex phenols in *Camellia* leaves by *Erotium* fungi [51].

Like other fungi, the *E. cristatum* INA01267 isolate is characterized by manifest hydrolase activity. It enables the fungus to grow on plant substrates at the expense of low molecular weight compounds formed by degrading biopolymers. Microbial fermentation resulted in significantly decreasing the fructose and sucrose contents in bay willow and apple leaf extracts and concomitantly changing the organic acid content. Lactic and citric acid accumulated in fermented bay willow leaves, while their contents in apple leaves, in contrast, drastically decreased. Overall, apple leaf fermentation is characterized by more complete carbohydrate oxidation and the production of lesser amounts of organic acids. In both kinds of herbal tea, post-fermentation brought about an increase in succinic acid content. Succinic acid possesses a wide range of adaptogenic properties, which is an important feature of functional beverages.

The amino acid composition of the original herbal teas is characterized by an elevated content of free amino acids in bay willow tea. Aspartic acid, threonine, valine, and alanine prevail. Apple leaves predominantly contain aspartic acid, serine, and alanine. Alterations in amino acid content after microbial fermentation indicate that plant protein compounds are hydrolyzed, and they do not suggest that the fungus preferentially grows on any specific amino acids. The levels of tyrosine and tryptophan that are utilized for synthesizing biogenic amines remained virtually unchanged in both plant substrates.

These results testify to a *homeostasis-maintaining strategy* used by the fungus with respect to the content of low molecular weight products of cell biopolymer hydrolysis on various plant substrates, including the leaves of bushes (*Camellia*), trees (*Malus*), and grasses (*Chamaenerion angustifolium*).

To reiterate, there is a substantial difference between the biological activity of post-fermented dark Chinese teas and that of microbially unfermented teas in terms of improving the operation of the immunity system, decreasing the activity of proinflammatory factors, and reducing the cholesterol level. This should provide an incentive for research and development

activities aimed at obtaining post-fermented herbal teas.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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